

A Cyclin D-Cdk4 Activity Required for G₂ Phase Cell Cycle Progression Is Inhibited in Ultraviolet Radiation-induced G₂ Phase Delay*

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Cyclin D-Cdk4 complexes have a demonstrated role in G₁ phase, regulating the function of the retinoblastoma susceptibility gene product (Rb). Previously, we have shown that following treatment with low doses of UV radiation, cell lines that express wild-type p16 and Cdk4 responded with a G₂ phase cell cycle delay. The UV-responsive lines contained elevated levels of p16 post-treatment, and the accumulation of p16 correlated with the G₂ delay. Here we report that in UV-irradiated HeLa and A2058 cells, p16 bound Cdk4 and Cdk6 complexes with increased avidity and inhibited a cyclin D3-Cdk4 complex normally activated in late S/early G₂ phase. Activation of this complex was correlated with the caffeine-induced release from the UV-induced G₂ delay and a decrease in the level of p16 bound to Cdk4. Finally, overexpression of a dominant-negative mutant of Cdk4 blocked cells in G₂ phase. These data indicate that the cyclin D3-Cdk4 activity is necessary for cell cycle progression through G₂ phase into mitosis and that the increased binding of p16 blocks this activity and G₂ phase progression after UV exposure.

Loss of normal cell cycle control mechanisms is a common theme in the development of most cancers, in particular the loss of mechanisms involved in sensing and repairing DNA damage. An increasing number of tumor suppressor genes have been found to be intimately involved in DNA damage responses, including cell cycle responses. The best documented of these are the tumor suppressor genes *TP53* and *Rb*. *TP53* is mutated in a high proportion of tumors, and its gene product (p53) is involved in cell cycle responses following DNA damage (1). p53 knockout mice develop relatively normally, but are prone to a range of tumors (2). *Rb* was identified as the retinoblastoma susceptibility gene, and its gene product (Rb) has a pivotal role in the late G₁ phase restriction point (3). Knockout of one allele of *Rb* in mice produced animals predisposed to pituitary tumors, but they did not develop retinoblastoma, and the homozygous knockout was embryonically lethal (4). These tumor suppressors exert their cell cycle effects by ultimately

modulating the levels and activities of the regulatory elements underlying cell cycle progression, the cyclin-dependent kinases.

The cyclin-dependent kinases are regulated in a complex manner, through cyclin subunit association; a series of tightly regulated phosphorylations and dephosphorylations (5); and the binding of inhibitory protein subunits, CKI proteins (Cdk inhibitors) (6). p53 can directly regulate the expression of one of these CKI proteins, p21 (1); and in response to DNA damage, p53-induced p21 expression is responsible for a G₁ phase delay (7). Fibroblasts from p21 knockout mice fail to delay in G₁ in response to similar damage (8). The mechanism by which Rb exerts its cell cycle inhibitory effects is somewhat complex, although this is largely via its ability to bind and inhibit the activity of a group of transcription factors (E2F) that are essential for progression into S phase and the expression of a number of positive and negative regulators of cell cycle progression (3). Thus, loss of Rb function would be predicted to result in a deregulation of entry into S phase. One of the cell cycle regulators whose expression is controlled by Rb function is itself a tumor suppressor, p16^{CDKN2A}. p16 is another CKI that acts specifically to inhibit cyclin D-Cdk4 and Cdk6, the cyclin-dependent kinases responsible for phosphorylating Rb during G₁ (3, 6). *CDKN2A* itself is mutated, deleted, or hypermethylated in a high proportion of human tumors (9). *CDKN2A* has also been identified as a melanoma susceptibility gene (10) and is deleted, mutated, or hypermethylated in a high proportion of melanoma cell lines (10).

Cell cycle responses to DNA damage induced by suberythral doses of UV radiation include both G₁ and G₂ phase delays. G₁ delays appear to be through a p53-dependent mechanism, with increased expression of p21 resulting in inhibition of G₁ cyclin/cyclin-dependent kinases (11–13). The G₂ delay is p53-independent and is via a block in the Cdc25-dependent activation of cyclin A-Cdk and cyclin B-Cdc2 complexes (14, 15). Cells undergoing the UV-induced G₂ delay appear to be identical to normal early G₂ phase cells, and in many respects, the delay appears to be a prolongation of that cell cycle state (15). The cells recover from the UV-induced block after a delay of 24 h and recommence normal proliferation (16). A major difference that has been noted between the UV-induced G₂-delayed cells and normally cycling cells at the equivalent cell cycle stage was a large increase in the level of p16 protein (17). Analysis of a number of mainly melanoma cell lines either deleted for *CDKN2A* or that expressed either mutant p16 or Cdk4 showed that only cells lines that expressed both wild-type p16 and Cdk4 displayed a UV-induced G₂ delay (16). Of the melanoma cell lines that had lost their UV-induced G₂ delay response, two expressed p16 with mutations that abolished Cdk4 (but not Cdk6) binding, and one line expressed the Cdk4 R24C muta-

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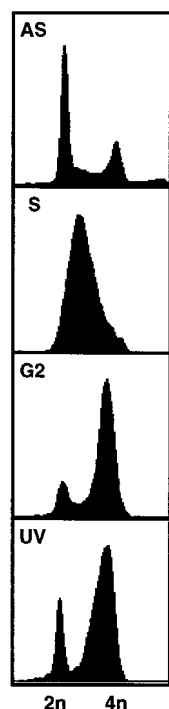


FIG. 1. Fluorescence-activated cell sorter profiles of HeLa cell cycle distribution of asynchronous (AS), thymidine block release synchronized S (S) and late S/ G_2 (G_2), and UV-irradiated cultures 24 h post-exposure (UV).

tion, which abrogated p16 binding (10, 16, 17). This suggested that the effects of p16 on the G_2 delay were being mediated through Cdk4, the most likely mechanism being p16 binding and inhibition of a cyclin D-Cdk4 activity. Here we show the existence of a cyclin D3-Cdk4 activity in late S/ G_2 phase cells that is inhibited in response to UV radiation through the increased association of p16 and provide evidence that this Cdk4 activity is necessary for normal G_2 phase cell cycle progression.

MATERIALS AND METHODS

Cell Culture and Metabolic Labeling—The HeLa (human cervical carcinoma) and human melanoma A2058 cell lines were cultured in RPMI 1640 medium supplemented with 5 and 10% Serum Supreme (BioWhittaker, Inc.), respectively. For synchrony experiments, the cell lines were blocked overnight with 2 mM thymidine and then released into fresh medium containing 24 μ M thymidine and deoxycytidine. S phase cell-enriched populations were obtained by harvesting at 4 h after release, and late S/ G_2 phase cell-enriched populations at 8 h after release. Mitotically delayed populations were collected when >30% of cells showed the rounded mitotic phenotype. Asynchronous cultures were irradiated with 10 J m⁻² UVC in 2 ml of prewarmed phosphate-buffered saline and then refed fresh medium. G_2 -delayed cells were obtained by harvesting at 24 and 20 h post-irradiation for HeLa and A2058 cells, respectively. The cell cycle status was confirmed by flow cytometry of the DNA content. See Fig. 1 for typical cell cycle profiles of the synchronized and UV-irradiated cultures.

Synchronized and UV-irradiated cells were metabolically labeled with 0.3 mCi/ml [³⁵S]-Promix (Amersham Pharmacia Biotech) in methionine- and cysteine-free medium for 2 h, chased in complete medium for the indicated times, washed twice with phosphate-buffered saline, and harvested. Synchronized late S/ G_2 phase cells or UV-irradiated cells (24 h post-irradiation) were metabolically labeled with 1 mCi/ml [³²P]_i in phosphate-free medium for 3 h or, in the case of caffeine release, labeled for 2 h and then a further 1 h after caffeine addition. Cells were washed twice with phosphate-buffered saline, harvested, and processed as described below for immunoprecipitation.

Immunoprecipitation and Immunoblotting—Cells were lysed in NETN buffer (100 mM NaCl, 1 mM EDTA, 0.5% Nonidet P-40, and 20 mM Tris, pH 8) supplemented with 150 mM NaCl, 5 μ g/ml aprotinin, 5 μ g/ml pepstatin, 5 μ g/ml leupeptin, 0.5 mM phenylmethylsulfonyl fluoride, 10 mM NaF, and 0.1 mM sodium orthovanadate. The cleared supernatants were immunoprecipitated using anti-p16 (17), anti-cyclin

A (Pharmingen), anti-cyclin B1 (18), or anti-Cdk4 or anti-Cdk6 (Santa Cruz Biotechnology) antibody with protein A-Sepharose. Immunoprecipitates from metabolically [³²P]- and [³⁵S]-labeled samples were resolved on 15% SDS-polyacrylamide gel and autoradiographed or quantitated by a PhosphorImager (Molecular Dynamics, Inc.). Other immunoprecipitates were immunoblotted for associated proteins using the indicated antibody. Cyclin D3 was detected using two specific monoclonal antibodies (Pharmingen G107-565 and Oncogene Research Products Ab 2).

Cdk4 immunoprecipitate Rb kinase assays were performed in a similar manner to that described elsewhere (19). Briefly, cell pellets (1–5 \times 10⁶ cells) were lysed in 50 mM Hepes, pH 7.5, 1 mM EDTA, 2.5 mM EGTA, 1 mM dithiothreitol, 250 mM NaCl, 10% glycerol, 0.1% Tween 20, 1 mM sodium fluoride, 0.1 mM sodium vanadate, and protease inhibitors as described above for 1 h on ice with occasional vortexing. The lysates were precleared with 30 μ l of a 50% suspension of protein A-Sepharose for 30 min, and the cleared supernatants were incubated with 1 μ g of anti-Cdk4 or anti-p16 antibody for 2–3 h and then precipitated by the addition of 20 μ l of a 50% suspension of the protein A-Sepharose for an additional 1 h. The precipitates were washed four times with the lysis buffer and then twice with the kinase buffer, and the kinase assay was performed with the addition of 30 μ l of reaction mixture containing 50 mM Hepes, pH 7.5, 10 mM MgCl₂, 50 mM ATP, 1 mM dithiothreitol, and 1–3 μ g of purified GST¹-Rb-(773–923) and 10 μ Ci of [³²P]ATP for 30 min. The reactions were stopped by the addition of SDS sample buffer and boiled for 2 min, and then the total reaction was separated on 10% SDS-polyacrylamide gel, stained with Coomassie Blue, dried, and autoradiographed. Cyclin B1 kinase assays were performed as described previously (15). The activity of the immunoprecipitated kinases was quantitated by phosphorimaging of the phosphorylated GST-Rb-(773–928) and histone H1 bands.

Transient Transfections—Expression constructs containing hemagglutinin (HA)-tagged wild-type Cdk4 and Cdk6 and the dominant-negative Cdk4 D158N and Cdk6 D163N mutants (10 μ g/10⁷ cells) (20) were transfected by electroporation into HeLa cells. Cdk4 and Cdk6 were cotransfected with mouse CDC37 (10 μ g/10⁷ cells) (21). Cells were harvested 48 h post-transfection, and overexpression of Cdk4 or Cdk6 was assessed by staining with anti-Cdk4 or anti-Cdk6 antibody detected with a fluorescein-labeled secondary antibody and DNA stained with propidium iodide and then analyzed by two-parameter flow cytometry (18). Cells expressing elevated levels of Cdk4 or Cdk6 were analyzed for their DNA content for cell cycle status.

Gel Filtration Analysis—Control unirradiated cells (either asynchronous or synchronized late S/ G_2 phase) or UV-irradiated G_2 -delayed cells were lysed in NETN buffer as described above, and the cleared supernatant (2 mg of protein) was applied to a Superose 12 column (Amersham Pharmacia Biotech) equilibrated in 20 mM Tris, pH 8, 2 mM EDTA, 1 mM dithiothreitol, and 150 mM NaCl. The column was developed by fast protein liquid chromatography (Amersham Pharmacia Biotech) at a flow rate of 0.25 ml/min, and 0.5-ml fractions were collected. Fractions were either immunoblotted for p16, Cdk4, Cdk6, or cyclin D3 or immunoprecipitated with anti-p16 and anti-Cdk4 antibodies and then analyzed by immunoblotting.

RESULTS

Increased p16-Cdk4 and p16-Cdk6 Complex Stability following UV Exposure—We have previously reported elevated levels of p16 in response to low dose UV radiation, which correlated with G_2 phase cell cycle delay (16, 17). Here we have investigated the consequences of p16 accumulation in two cell lines: HeLa cells, which are human papilloma virus-transformed epithelial cells derived from an adenocarcinoma, and A2058 cells, derived from a malignant melanoma. The following experiments were conducted in both cell lines with essentially identical results, and consequently, only the data obtained with HeLa cells are shown in most cases.

To identify proteins associated with p16 in HeLa and A2058 cells, [³⁵S]Met/Cys-labeled cells were lysed, and p16-associated complexes were immunoprecipitated and analyzed by SDS-polyacrylamide gel electrophoresis. Three bands were specifically precipitated with the antibody in both control and UV-

¹ The abbreviations used are: GST, glutathione S-transferase; HA, hemagglutinin.

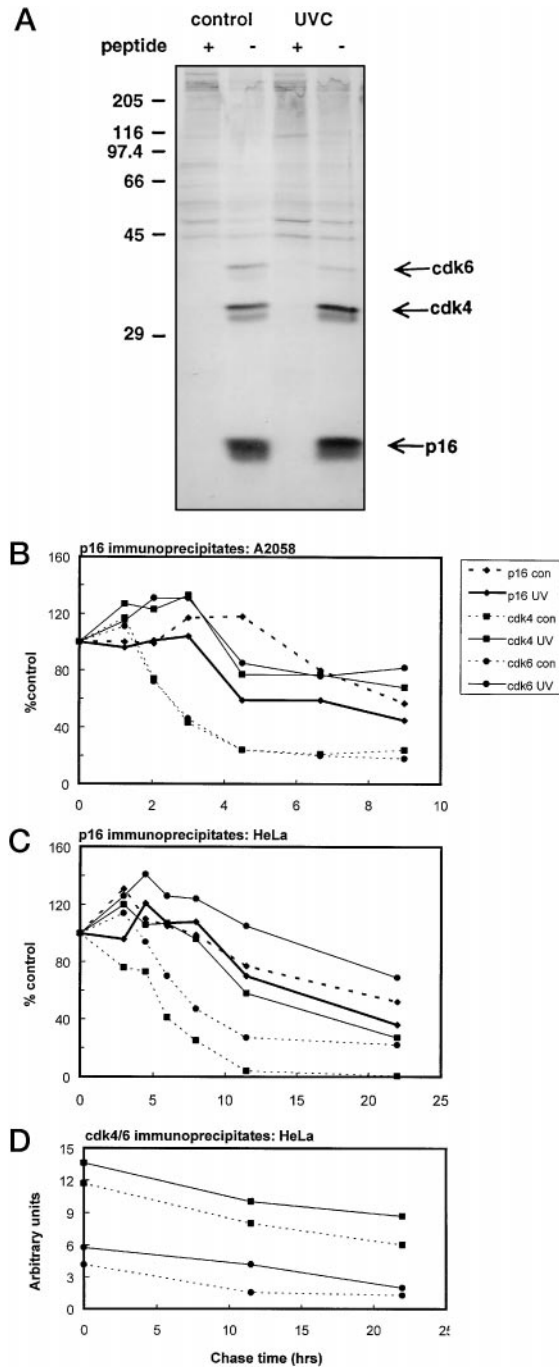


FIG. 2. UV radiation increases the stability of the p16-Cdk4 and p16-Cdk6 complexes. *A*, lysates of either control asynchronous or UV-irradiated G₂-delayed HeLa cells metabolically labeled with [³⁵S]methionine and cysteine were immunoprecipitated with either anti-p16 antibody or antibody preincubated with the immunogen peptide, and the immunoprecipitates were analyzed on 15% SDS-polyacrylamide gel. The positions of p16, Cdk4, and Cdk6 are indicated. *B* and *C*, shown are the relative levels of ³⁵S-labeled p16, Cdk4, and Cdk6 associated with p16 immunoprecipitates during a pulse-chase experiment of labeled A2058 and HeLa cultures. *D*, shown are the levels of ³⁵S-labeled Cdk4 and Cdk6 immunoprecipitated with their respective antibodies from the same HeLa pulse-chase experiment as *C*. The intensity of the labeled bands is in arbitrary units. *con*, control.

irradiated HeLa cells (Fig. 2A), which were identified by immunoblotting as p16, Cdk4, and Cdk6 (Fig. 3A). No other ³⁵S-labeled bands were found to be specifically associated with p16 in either control or UV-irradiated samples. p53 accumulation following DNA damage involves an increase in p53 protein stability (22), and ³⁵S pulse-chase experiments were performed

to investigate whether the UV-induced accumulation of p16 utilized a similar mechanism. There was little difference in the half-life of p16 in asynchronously growing and UV-irradiated G₂-delayed A2058 and HeLa cultures, although p16 had a shorter half-life in A2058 cells (6 h; *n* = 1) compared with HeLa cells (9.5 ± 0.5 h; *n* = 3) (Fig. 2, *B* and *C*). Unexpectedly, the half-life of Cdk4 and Cdk6 associated with the p16 immunoprecipitates increased markedly (2–3-fold) in the UV-irradiated cells compared with unirradiated controls in both cell lines. The half-life of the p16-Cdk4 complex increased from 5 ± 1 h in the control HeLa cells to 12 ± 1 h in the UV-irradiated cells (*n* = 3). The increased half-life of the p16-Cdk6 complex was similar, although the very low levels of Cdk6 in HeLa cells precluded accurate estimates. The increased half-life of p16-Cdk4 and p16-Cdk6 in control cells compared with irradiated A2058 cells was similar, from 2 to 3 h to 10 to 14 h, respectively. This was not due to any increase in the half-life of the Cdk4 and Cdk6 proteins themselves (Fig. 2C), but suggested a decrease in the rate of exchange of newly synthesized p16-bound Cdk4 and Cdk6 with the unlabeled pools of Cdk4 and Cdk6, reflecting an increase in the stability of the p16-Cdk4 and p16-Cdk6 complexes following UV radiation. Although asynchronously growing cultures were used as controls in these experiments, pulse-chase data from synchronized G₂ cell-enriched cultures gave essentially the same results (data not shown).

UV Radiation Increases p16 Association with Cdk4 and Cyclin D—The association of p16 with Cdk4 and Cdk6 was further investigated by immunoblotting. In HeLa cells, the levels of Cdk4 and Cdk6 were unchanged throughout the cell cycle or following UV radiation, whereas p16 levels increased >4-fold following UV radiation (Fig. 3A), as shown previously (16, 17). The sole cyclin D partner for Cdk4 in HeLa cells, cyclin D3,² was also expressed at a constant level in these samples. The very low level of cyclin D3 associated with Cdk4 was confirmed by immunoblotting with two separate anti-cyclin D3 monoclonal antibodies (see “Materials and Methods”). When Cdk4 immunoprecipitates from these same cell lysates were immunoblotted for cyclin D3, no change in the level of this protein was apparent, whereas the level of p16 associated with Cdk4 increased in the UV-irradiated sample, paralleling the increase in total p16 levels (Fig. 3B). Immunoblotting of p16 immunoprecipitates from similar lysates also showed constant levels of cyclin D3 associated with p16 in each sample and no change in the Cdk4 levels associated with p16 in the UV-irradiated cells (Fig. 3C), in contrast to the Cdk4-associated p16 levels (Fig. 3B).

To further investigate the p16-Cdk4-cyclin D3 complexes, UV-irradiated and control HeLa cultures were lysed and fractionated on a Superose 12 gel filtration column, and the fractions were immunoblotted for p16, Cdk4, Cdk6, or cyclin D3. p16 eluted as two distinct peaks; the level of p16 eluting in the higher *M_r* peak increased >4-fold in the UV-irradiated sample compared with the control, whereas the increased level of p16 in the lower molecular weight peak was more modest, ~2-fold (Fig. 4, *A* and *B*). Cdk4 eluted as a broad peak from fractions 13 to 20, and Cdk6 eluted as a tighter peak around fraction 15, whereas the majority of the cyclin D3 was associated with very high molecular weight complexes, peaking in fraction 4, although very low levels were detected through to fraction 14 (data not shown). The distribution of Cdk4, Cdk6, and cyclin D3 did not change discernibly between control (either asynchronous or synchronized late S/G₂ phase cultures) and UV-irradiated samples. The p16 and Cdk4 complexes in these fractions were analyzed by sequential immunoprecipitation

² X. Q. Wang and B. G. Gabrielli, unpublished observations.

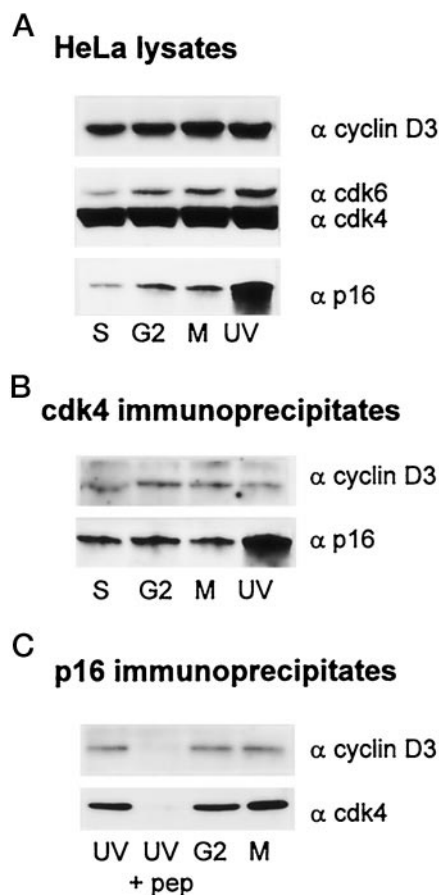


FIG. 3. Level of p16 associated with Cdk4 increases following UV exposure. Shown are the levels of cyclin D3, Cdk4, Cdk6, and p16 in total lysates (A), associated with anti-Cdk4 (B) or anti-p16 (C) immunoprecipitates, and from synchronized or UV-irradiated HeLa cell cultures. The specificity of the Cdk4 and cyclin D3 association with p16 was demonstrated by preincubating anti-p16 antibody with the immunogen peptide (UV + pep).

with anti-p16 antibody under conditions that immunodepleted p16 and then immunoprecipitated with anti-Cdk4 antibody to examine the Cdk4 complexes not containing p16. Both p16 and Cdk4 immunoprecipitates were immunoblotted for p16, Cdk4, and cyclin D3. p16 immunoprecipitates of the gel filtration fractions revealed that p16 was present in three different complexes: with cyclin D3 and Cdk4 (fractions 13–15), a complex with Cdk4 only (fractions 17–19) (Fig. 4, C and D), and a monomer, as p16 immunoprecipitates from fractions 20–22 contained no Cdk4 or Cdk6 (data not shown). The level of cyclin D3, Cdk4, and p16 detected in the p16 immunoprecipitate from fraction 13 was increased 2-fold in the UV-irradiated sample compared with the late S/ G_2 phase control (Fig. 4, C and D). Reciprocal Cdk4 immunoprecipitation and p16 immunoblotting of equivalent fractions in a separate experiment revealed the same increase of the p16-Cdk4-cyclin D3 complex in UV-irradiated samples (data not shown). Cdk4 immunoprecipitates from the p16-immunodepleted fractions (demonstrated by the absence of detectable p16 in the Cdk4 immunoprecipitates) (Fig. 4C) revealed a higher level of cyclin D3 associated with Cdk4 (fractions 13 and 15) in the control compared with the irradiated samples. The relative intensity of the Cdk4 and cyclin D3 bands in the p16 and Cdk4 immunoprecipitates indicated that the majority of Cdk4 and cyclin D3-Cdk4 was associated with p16, with only small pools of cyclin D3-Cdk4 and Cdk4 (fraction 17, anti-Cdk4 immunoprecipitate) (Fig. 4C) not associated with p16, and that these free pools were reduced following UV exposure (Fig. 4C).

In an effort to identify novel p16-containing complexes in either control or UV-irradiated cells, earlier eluting, higher molecular weight fractions (fractions 1–4 and 5–8) were pooled, and the presence of p16 was examined by immunoprecipitating with anti-p16 antibody from the pooled fractions and immunoblotting with the same antibody. No p16 was detected in the earlier eluting fractions from either control or irradiated samples.

A Cdk4 Activity in Late S/Early G_2 Phase Is Inhibited following UV Radiation—The previous experiments showed that a consequence of the increased p16 levels in the UV-irradiated cells was an increase in the p16-Cdk4-cyclin D3 complex. This suggested that cyclin D3-Cdk4 may be active in the late S/early G_2 phase of normally cycling cells and inhibited in the UV-irradiated G_2 -delayed cells. The presence of such an activity was assessed in HeLa cell cultures synchronized using a thymidine block/release protocol. Cells were collected at times up to 14 h post-release as they progressed from G_1 /S through mitosis into the next G_1 phase (Fig. 5). Cdk4 activity at each time point was examined using a Cdk4 immunoprecipitate kinase assay with GST-Rb-(733–928) as a substrate. A kinase activity associated with the Cdk4 immunoprecipitates was detected as cells commenced entering G_2 phase (6 h), peaked at 8 h, and then decreased again as the cells existed mitosis (11 h). The peak of activity correlated with the shoulder of p9^{CKSHs1}-Sephacryl-S4B kinase activity in G_2 , prior to the major peak of H1 kinase activity in mitosis (10 h). The immunoprecipitated kinase activity was shown to be due to a Cdk4 complex, as nonreactive serum or immune peptide-blocked anti-Cdk4 antibody immunoprecipitated only background Rb kinase activity from late S/ G_2 phase HeLa cell lysates, and no phosphorylated bands were detected in immunoprecipitates assayed without addition of the Rb substrate (Fig. 6A). Furthermore, the Cdk4-associated activity was not inhibited by 50 μ M olomoucine, a strong inhibitor of Cdk2 and Cdc2 kinases, but relatively ineffective against Cdk4 (23), which resulted in 80% inhibition of immunoprecipitated Cdk2 activity (Fig. 6, A and B). When the Cdk4 activity in UV-irradiated G_2 -delayed samples was tested, it was found to be inhibited in both HeLa and A2058 cells when compared with the respective cell cultures enriched for S, late S/ G_2 , and M phase cells (Fig. 6C). In HeLa cells, Cdk4 activity was decreased by 60% in the UV-irradiated cells compared with the G_2 cell-enriched cultures (Fig. 6D).

Activation and Phosphorylation of Cdk4 Are Correlated with Exit from UV-induced G_2 Delay—We have previously shown that the UV-induced G_2 phase delay results in a block in the Cdc25-dependent activation of the mitotic cyclin-Cdk complexes in G_2 and M (15). Here we have demonstrated that the normal activation of cyclin D3-Cdk4 in early G_2 phase was inhibited during the UV-induced G_2 delay and that cyclin D3-Cdk4 activation occurred in G_2 prior to the activation of cyclin B-Cdc2 at the G_2 /M transition (Fig. 7A). This suggested a possible causal relationship between cyclin D3-Cdk4 activation and the activation of the cyclin-Cdk complexes later in G_2 . This relationship was examined in UV-irradiated HeLa cells induced to progress through the G_2 delay into mitosis with 5 mM caffeine (24), and the activities of Cdk4 and cyclin B1 kinases were measured. Addition of caffeine resulted in a rapid exit from the G_2 delay, and cells commenced accumulating in G_1 by 2 h following caffeine addition (Fig. 7B). Cdk4 activity was almost maximal within 1 h following caffeine addition to the culture medium and then started to decline by 3 h, whereas cyclin B1-Cdc2 activity was not maximal until 3 h following caffeine addition (Fig. 7C). Thus, the block of Cdk4 activation was relieved by the addition of caffeine, was correlated with

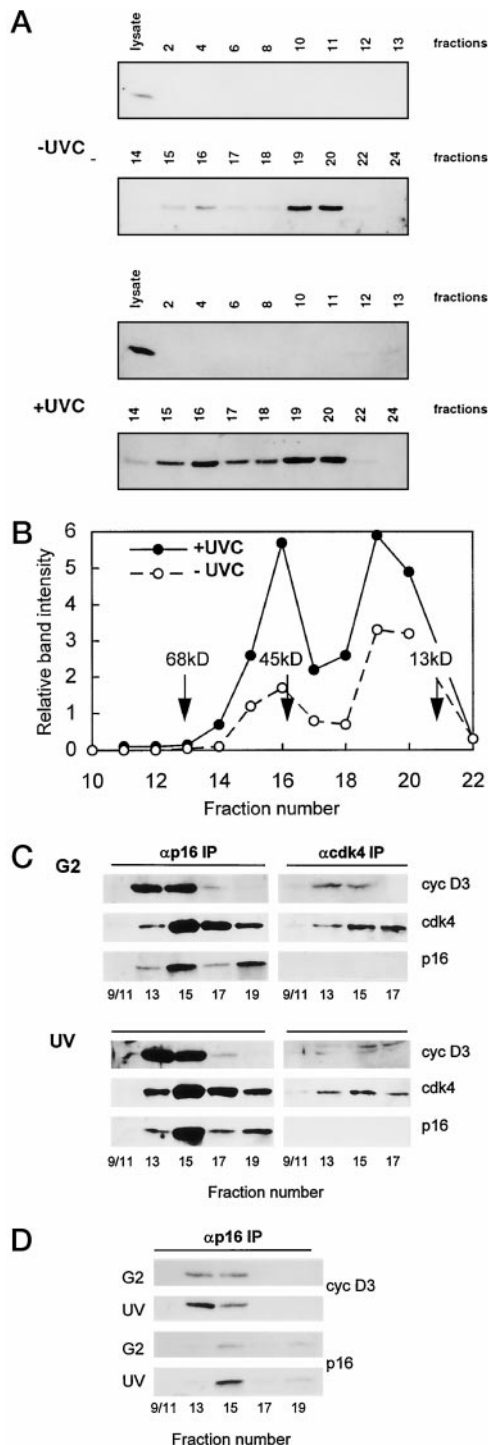


FIG. 4. Increased levels of p16 bound to cyclin D3-Cdk4 complexes after UV exposure. Asynchronous control ($-UVC$) or UV-irradiated G_2 -delayed ($+UVC$) HeLa cell lysates were fractionated on a Superose 12 gel filtration column, and the indicated column fractions were immunoblotted for p16 (A). The level of p16 in the fractions was quantitated by laser densitometry of the immunoblots (B). In C, control synchronized late S/G_2 phase (G_2) or UV-irradiated (UV) HeLa cell lysates were fractionated as described for A. Equivalent fractions were sequentially immunoprecipitated with anti-p16 and then anti-Cdk4 antibodies, and the immunoprecipitates (IP) were immunoblotted for cyclin D3 (*cyc D3*), Cdk4, and p16. The anti-p16 and anti-Cdk4 immunoprecipitates were exposed together for the same period to demonstrate the relative levels of Cdk4 and cyclin D3 complexed with p16 and not associated with p16. The upper band in the UV-treated cyclin D3 blot is cross-reactive IgG heavy chain. The shorter exposure of the immunoblots shown in C shows the relative increase in cyclin D3 and p16 levels in anti-p16 immunoprecipitates from UV compared with control samples (D).

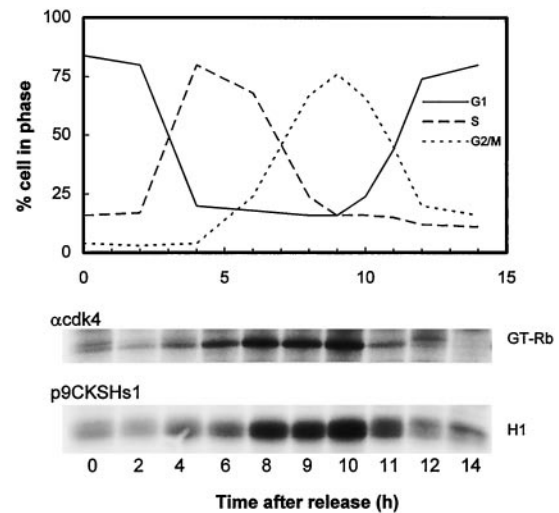


FIG. 5. A Cdk4 activity is present in normally cycling late S/G_2 phase cells. HeLa cells were synchronized in early S phase with a thymidine block and then released. Cells were harvested at the indicated times after release and analyzed for cell cycle status by flow cytometry and for Cdk4 activity by Cdk4 immunoprecipitate kinase assay using GST-Rb-(773-928) (*GT-Rb*) as substrate or for Cdk2 and Cdc2 kinase activity precipitated by p9^{CKSHs1}-Sepharose using histone H1 as substrate.

continued progression through G_2 , and preceded cyclin B1-Cdc2 activation at the G_2/M transition.

We earlier demonstrated that the level of p16 associated with Cdk4 increased in UV-irradiated cells (Fig. 3B). The binding of p16 to Cdk4 complexes after caffeine release was examined to see whether the activation of Cdk4 correlated with decreased p16 association. By 1 h post-caffeine addition, the increased level of p16 associated with Cdk4 complexes in the UV-irradiated cells had returned to control levels, correlating with the activation of cyclin D3-Cdk4 (Fig. 7, C and D). The levels of p16 did not change discernibly during this time (data not shown) (16), indicating that the reduced binding of p16 is not simply due to reduction in the level of p16.

The phosphorylation status of Cdk4 was examined to determine whether the activation of Cdk4 might involve a phosphorylation event. Cdk4 immunoprecipitates from metabolically $^{32}P_i$ -labeled HeLa cells, either UV-irradiated G_2 -delayed cells or at 1 h post-caffeine addition, revealed a 1.8-fold increase in the phosphorylation of Cdk4 (Fig. 8A). Interestingly, p16 immunoprecipitates from equivalent samples did not contain any labeled bands, although immunoblotting of 10% of these immunoprecipitates for Cdk4 showed that all contained similar levels of Cdk4 protein (data not shown). In a separate experiment, a 2-fold difference in the phosphorylation state of Cdk4 was observed in Cdk4 immunoprecipitates from UV-irradiated and synchronized late S/G_2 cells (Fig. 8B). Again, no labeled bands were detected in anti-p16 immunoprecipitates. No phosphorylation of p16 was detected under any condition.

Cdk4 Activity Is Required for Progression through G_2 into Mitosis—The data presented suggest a role for Cdk4 activity in cell cycle progression through early G_2 into mitosis. To directly test whether Cdk4 is involved in cell cycle progression, the dominant-negative Cdk4 D158N mutant was overexpressed in HeLa cells, and the cell cycle status of the overexpressing population was assessed by flow cytometry. Experiments using the equivalent mutants of Cdc2 and Cdk2 have demonstrated the cell cycle transitions regulated by these cyclin-dependent kinases (20). To obtain sufficiently high levels of Cdk4 overexpression for the mutant to exert a dominant-negative effect, the Cdk4 was cotransfected with mouse CDC37, which increased

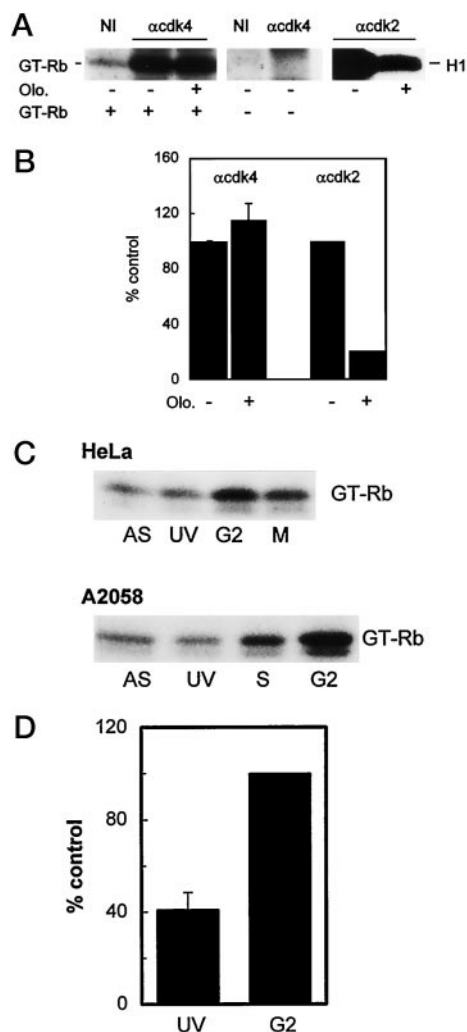


FIG. 6. G₂ phase Cdk4 activity is inhibited in UV-irradiated G₂-delayed cells. *A*, late S/G₂ phase HeLa cell lysates were immunoprecipitated with nonimmune serum (NI) or anti-Cdk4 or anti-Cdk2 antibody, and the immunoprecipitates were assayed either with or without substrate and 50 μ M olomoucine (Olo.) as indicated. *B*, the level of Cdk4 and Cdk2 immunoprecipitate activity is expressed as a percentage of the minus-olomoucine control. *C*, Cdk4 immunoprecipitate Rb kinase activity from the indicated synchronized and UV-irradiated HeLa and A2058 cells is shown. *D*, the Cdk4 Rb kinase activity in UV-irradiated or control late S/G₂ phase HeLa cells is expressed as a percentage of the control from four separate experiments. GT-Rb, GST-Rb(773–928).

the stability of the overexpressed Cdk4 protein (21). Cotransfection of Cdk6 with CDC37 had little effect on Cdk6 expression, as reported previously (21). Immunoblotting for Cdk4 and Cdk6 revealed that wild-type and mutant versions of both proteins were strongly expressed, with the ectopically expressed proteins being distinguishable from the endogenous forms due their slightly retarded electrophoretic mobility, a result of the HA tag (Fig. 9A). Cells overexpressing Cdk4 and Cdk6 were discriminated on the basis of increased Cdk4 or Cdk6 staining using flow cytometry, and the cell cycle distribution of the overexpressing population was determined. The wild-type Cdk4-overexpressing population showed a small increase in the G₂/M population compared with control vector-transfected cells, but the mutant-overexpressing cells showed a 1.5-fold accumulation of G₂/M phase cells over the wild type-overexpressing cells (Fig. 9B). Inspection by immunofluorescent microscopy of transfected cells stained for ectopic Cdk4 expression (HA tag) and endogenous cyclin B1 showed that a higher proportion of cells strongly overexpressing the Cdk4

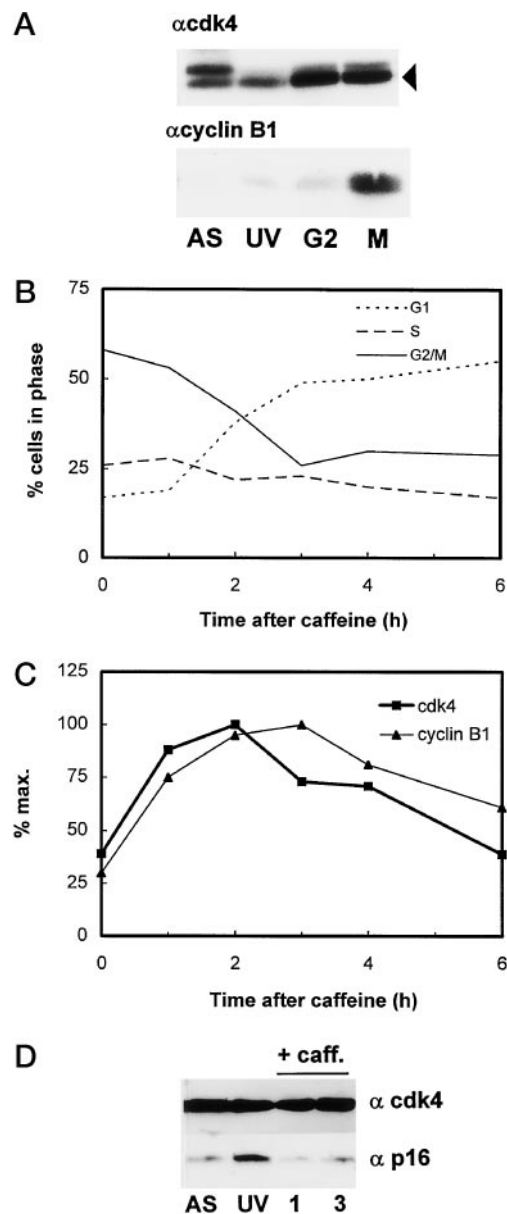


FIG. 7. Caffeine-induced release from the G₂ phase delay is correlated with increased Cdk4 activity and decreased p16 binding. *A*, shown is the cyclin B1 and Cdk4 immunoprecipitate kinase activity from asynchronous (AS), UV-irradiated G₂-delayed (UV), late S/G₂ (G2), and mitotically enriched (M) HeLa cells. Phosphorylated GST-Rb(773–928) is indicated (arrowhead). *B*, UV-irradiated G₂-delayed HeLa cultures were treated with 2 mM caffeine (time 0), the cells were harvested at the indicated times, and their DNA content was analyzed by flow cytometry for cell cycle status. *C*, lysates from equivalent samples were assayed for Cdk4 and cyclin B1 immunoprecipitate kinase activity (*C*). The activities are expressed as a percentage of their peak activity. *D*, the level of p16 bound to Cdk4 from asynchronous, UV-irradiated G₂-delayed, and UV-irradiated cultures either 1 or 3 h post-caffeine (caff.) addition was assessed by immunoblotting anti-Cdk4 immunoprecipitates for Cdk4 (as a loading control) and p16.

mutant contained high levels of cytoplasmic cyclin B1, an indicator of G₂ phase (25), compared with the untransfected population, indicating that mutant Cdk4 overexpression resulted in accumulation of G₂ phase cells. As a further control for the specificity of Cdk4 action, wild-type and dominant-negative mutant forms of Cdk6 were also transfected into HeLa cells. Cells overexpressing either form of Cdk6 produced a cell cycle profile similar to that produced by the wild-type Cdk4-overexpressing cells. Thus, the increased accumulation of G₂ phase cells observed with mutant Cdk4 overexpression was due

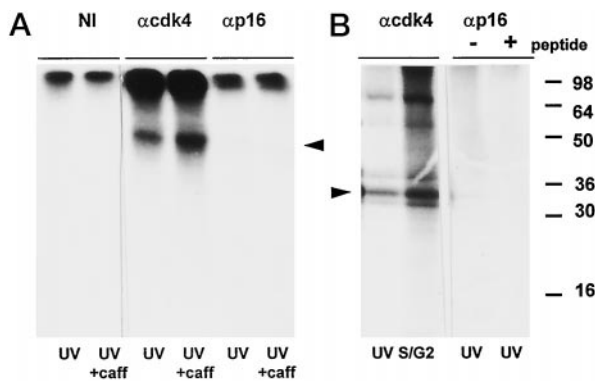


FIG. 8. UV exposure decreases the phosphorylation of Cdk4. *A*, UV-irradiated HeLa cells 22 h post-exposure were labeled for 2 h with $^{32}\text{P}_i$ in phosphate-free medium; 5 mM caffeine (*caff*) was added to half the plates and incubated an additional 1 h, and the cells were then harvested. Lysates prepared from these samples were initially incubated with nonimmune serum (*NI*) and then either anti-Cdk4 or anti-p16 antibody. The immunoprecipitates were washed and analyzed on 15% SDS-polyacrylamide gel. The position of ^{32}P -labeled Cdk4 is indicated (arrowhead). *B*, cultures of UV-irradiated cells 22 h post-exposure and synchronized late S/ G_2 phase cells containing similar numbers of cells were labeled for 3 h and processed as for *A*. In this experiment, the UV sample was incubated with anti-p16 antibody or with anti-p16 antibody preincubated with the p16 peptide to block immunoreactivity (see Fig. 2*A*). The positions of molecular weight markers ($\times 10^3$) are shown, and the ^{32}P -labeled Cdk4 band is indicated (arrowhead).

to the specific inhibition of a Cdk4-dependent activity and provides further evidence for a Cdk4-dependent step in G_2 phase cell cycle progression.

DISCUSSION

Cyclin D-Cdk4 complexes have been reported to exist throughout the cell cycle in many cell types (26, 27), although the function of these complexes through the cell cycle, apart from regulating the Rb-dependent G_1 checkpoint, is unknown. Here we have demonstrated that cyclin D3-Cdk4 complexes are present throughout the cell cycle and that this complex is activated in late S/early G_2 phase in both HeLa and A2058 cells. The presence of a Cdk4 activity in G_2 phase was first indicated by Matsushime *et al.* (27), who showed, in mouse fibroblasts overexpressing Cdk4 and cyclin D, peaks of Cdk4 activity both in G_1 and G_2 cell-enriched fractions. In the cell lines tested here, little G_1 phase Cdk4 activity was detected (the low level of Cdk4 activity immunoprecipitated from lysate of asynchronous cultures represents Cdk4 activity from $>60\%$ G_1 phase cells). The absence of G_1 phase Cdk4 activity in Rb-defective cell lines, a consequence of human papilloma virus-E7 expression in HeLa cells and loss of Rb expression in A2058 cells,² has been reported by others (28, 29).

The high levels of heterodimeric p16-Cdk4 and p16-Cdk6 complexes and monomeric p16 detected in HeLa and A2058 cells are also a common feature of Rb-defective cell lines (30, 31). In both HeLa and A2058 cells, only a small proportion of Cdk4 associated with cyclin D3, in agreement with other studies that showed that high levels of p16 expression reduce cyclin D binding to Cdk4 (30, 31).

In response to UV exposure, p16 association with Cdk4 is elevated, presumably a result of both increased p16 levels and the increased stability of p16-Cdk4 and p16-Cdk6 complexes. A clear consequence of the increased association of p16 with Cdk4 complexes is the inhibition of cyclin D3-Cdk4 activity. This was correlated with decreased phosphorylation of Cdk4 compared with either normal early G_2 phase cells or cells released from the UV-induced block with caffeine. The phosphorylation detected is likely to be at the activating Thr-172 on Cdk4 catalyzed by CAK (32), which has been demonstrated to be blocked

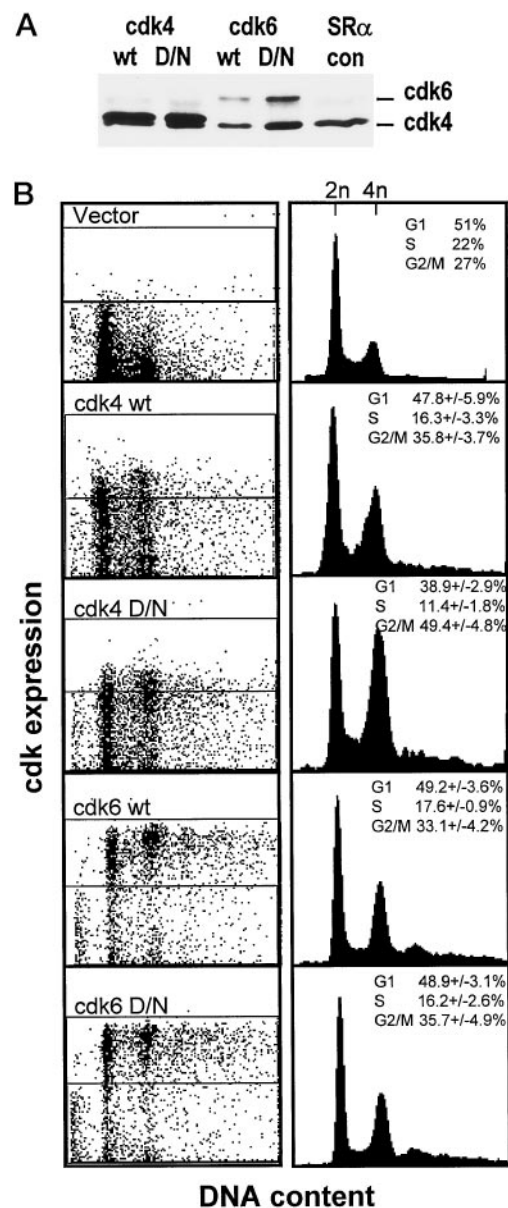


FIG. 9. Overexpression of a dominant-negative form of Cdk4 produces a G_2 phase delay. *A*, HeLa cells were transiently transfected with either a control vector (*SRα con*) or vector containing the indicated HA-tagged wild-type (*wt*) or dominant-negative mutant (*D/N*) Cdk4 or Cdk6 cDNA. Cells were harvested and immunoblotted with anti-Cdk4 and anti-Cdk6 antibodies. The ectopically expressed proteins are distinguished from the endogenous versions by their slower electrophoretic mobility due the HA tag. *B*, cells transfected as described for *A* were stained with either anti-Cdk4 or anti-Cdk6 antibody detected with a fluorescein-labeled secondary antibody and with propidium iodide for DNA. The Cdk4- and Cdk6-overexpressing cells were gated as the cells with elevated fluorescein signal over the empty vector control levels (Cdk expression), and the DNA content of the gated overexpressing population (population above the cutoff on dot plots on the left) is shown in the histograms on the right. The vector control is the total DNA histogram. The percentages of cells in each cell cycle stage are mean \pm S.D. from three separate experiments.

by p16 *in vitro* (33). The absence of phosphorylated Cdk4 in p16 immunoprecipitates fits well with the proposal that the increased p16 association is inhibiting Thr-172 phosphorylation of Cdk4 and thereby blocking its activation.

The observed UV-induced increase in the stability of the p16-Cdk4 and p16-Cdk6 complexes is unexpected. A recent mutational analysis of Cdk4 examining residues involved in p16 binding may provide a mechanism by which this increased

complex stability is acquired. This study demonstrated that mutation of Thr-172 to the nonphosphorylatable Ala residue increased p16 binding >2-fold (34). The crystal structure of the p16-Cdk6 complex revealed the T-loop containing Thr-172 to lie close to the first ankyrin repeat of p16 (35), and the presence of a highly charged phosphoryl group at Thr-172 is likely to alter the binding avidity of p16. Our findings that increased p16 binding correlated with decreased Cdk4 phosphorylation in UV-irradiated cells and that decreased p16 binding following caffeine-induced release from the G₂ block correlated with increased Cdk4 phosphorylation support a mechanism where a decrease in Cdk4 Thr-172 phosphorylation may increase p16 binding, which in turn will block the rephosphorylation of Thr-172 by CAK. It is not clear how caffeine changes the balance of phosphorylation and p16 binding so rapidly, and this whole mechanism will require further study.

The decreased phosphorylation of Cdk4 during the G₂ delay following UV exposure, in particular the absence of any phosphorylation of p16-bound Cdk4, differentiates this Cdk4-dependent cell cycle block from a Cdk4-dependent G₁ phase block also initiated by low dose UV radiation (36). It was demonstrated that Cdk4 was phosphorylated at Tyr-17 in response to UV radiation of cells in early G₁ phase and that this blocked G₁ phase progression (36). However, this mechanism appeared to be specific for the G₁ block, and cells expressing wild-type Cdk4 or a nonphosphorylatable Cdk4 Y17F mutant were equally susceptible to DNA damage and displayed similar survival when irradiated as they entered S phase, suggesting that later cell cycle checkpoints did not utilize this mechanism.

A lot of attention has been given to the role of cyclin D-Cdk4 complexes in regulating the G₁ phase Rb function, and there is currently little evidence of a role for these complexes later in the cell cycle. Much of the work on cyclin D has focused on cyclin D1, and although there is some degree of functional redundancy between the D-type cyclins, it becoming evident that all D-type cyclins are not equivalent. For example, overexpression of cyclin D1 (but not cyclin D3) accelerated G₁ progression in fibroblasts (27), and overexpression of cyclins D2 and D3 (but not cyclin D1) inhibited granulocyte differentiation (37). In estrogen-stimulated MCF-7 breast cancer cells, cyclin D1 transcript and protein levels correlated with G₁ phase progression, whereas expression of cyclin D3 message and protein was delayed, with cyclin D3 protein reaching maximal levels as cells progressed into G₂ phase (19). Substrate specificity differences have also been found between the cyclin D1-Cdk4 and D3-Cdk4 complexes *in vivo* and *in vitro* (38). Thus, it is likely that different cyclin D-Cdk4 complexes may have distinct functions, dependent on the cell type in which they are expressed and the timing of their expression and activation during the cell cycle.

The existence of a G₂ phase cyclin D-Cdk4 activity would also imply a regulatory mechanism to control this activity. The constitutive presence of the cyclin D3-Cdk4 complex throughout the cell cycle precludes cyclin-Cdk complex formation; thus, activity must be regulated at the level of phosphorylation and/or CKI interaction, which may act in concert to ensure very tight regulation. Agents or conditions that increase CKI levels at a particular point in the cell cycle presumably utilize these inhibitors to block cell cycle progression beyond that point until this inhibition is relieved. There are many reports demonstrating that overexpression of p16 inhibits cyclin D-Cdk4-dependent phosphorylation of Rb and G₁ phase progression (29, 39, 40) and proliferation in an Rb-dependent manner (29, 41, 42). Small increases in endogenously expressed p16 protein were found as serum-stimulated cells entered S phase (43) and at the S/G₂ transition in synchronized HeLa cells (17), although these

were not correlated with discernible cell cycle delays. Much larger increases in p16 levels have been found in senescing cells, correlating with inhibition of the G₁ and S phase cyclin/cyclin-dependent kinases (44), and as a delayed response to low doses of UV radiation, correlating with a G₂ delay (16, 17) and inhibition of cyclin D3-Cdk4 (this report). The UV-induced elevated p16 levels appear to be a specific response to UV-induced damage, as other genotoxic agents that produce a G₂ delay in HeLa and A2058 cells, *e.g.* cisplatin and ionizing radiation, do not affect p16 protein levels.³ Interestingly, a recent report has provided evidence for a p16-dependent checkpoint in G₁ in response to a wide variety of DNA-damaging agents in p53-deficient cell lines (45). This checkpoint is dependent on Rb and Cdk4, although the levels of p16 appeared unchanged in this response, differentiating it from the UV response investigated here, which appears to be independent of Rb function.

The data reported here provide evidence for a cyclin D-Cdk4 activity in late S/G₂ phase that appears to be required for normal cell cycle progression through G₂ phase into mitosis. This Cdk4 activity is inhibited in response to UV radiation, and inhibition of this activity appears to be involved in the G₂ phase delay observed. It will be of interest to see whether this role for p16 in a G₂ checkpoint is UV-specific or a more general cell cycle checkpoint control. It may also provide a mechanistic basis for the strong genetic and epidemiological evidence linking UV radiation, p16^{CDKN2A} function and melanoma.

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**A Cyclin D-Cdk4 Activity Required for G₂ Phase Cell Cycle Progression Is Inhibited
in Ultraviolet Radiation-induced G₂ Phase Delay**

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